Histological Architecture of the Nasal Region in *Rana chensinensis*

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Abstract The nasal region of *Rana chensinensis* is divided into the nasal capsules and nasal cavities. In this study, we investigated the adult histological structure of the nasal capsules and nasal cavities in the frog *R. chensinensis* under the microscope. We found that an eminentia olfactoria is present in this frog and the presence of the eminentia olfactoria may be connected to a terrestrial life style. The double staining method using alcian blue and alizarin red showed that the septomaxilla, the most important bone associated with the olfactory capsules, is an intramembranous bone in *R. chensinensis*. The opening of the nasolacrimal duct showed a close proximity to the apertura nasalis externa. The presence of the nasolacrimal duct in the olfactory region may be an adaptation to a terrestrial environment. The function of the vomeronasal and olfactory organs is discussed in the paper.

Keywords frog, *Rana chensinensis*, olfactory organ, vomeronasal organ, skeletal morphology

1. Introduction

The olfactory system is subdivided mainly into the olfactory system and the vomeronasal system. Nearly all vertebrates possess an olfactory organ (Eisthen, 1997). However, the presence or absence of the vomeronasal organ varies among vertebrates. The olfactory organ mainly samples volatile substances, while the vomeronasal organ is commonly assumed to be specialized for detecting pheromones (Wang *et al.*, 2008; Taniguchi *et al.*, 2011). Generally, the vomeronasal organ first evolved in amphibians (Eisthen, 1992). There is considerable controversy on whether the vomeronasal organ is a water or air sampling organ. Broman (1920) discovered that the vomeronasal organ in amphibians is homologous with the olfactory organ in fishes. The olfactory organ arose later as an adaptation to terrestrial life while the vomeronasal organ sensed water-borne substances. On the contrary, Bertmar (1981) insisted that the vomeronasal organ is present only in tetrapods but is absent in fish, and the organ first begins to detect odorants in air.

Many authors have described the morphology of the olfactory organ and the bones of the olfactory region in anurans. For example, Higgins (1920) published the earliest study on the olfactory organs of the bufonids; then, Michael (1961) published a description of the bone of the olfactory organs in *Bufo regularis* Reuss; and recently, Pugener and Maglia (2007) have conducted similar studies on the nasal development of *Spea*. They used vector graphic displays for their drawings to show the morphological characters of the olfactory region in detail. Moreover, references to the adult cranial morphology of other species have been found in a number of publications. Though the exhaustive studies on the cranial morphology of salientian species have been done, little attention has been devoted to *R. chensinensis*. In particular, little is known of the function of the nasolacrimal duct in the olfactory system. The goal of this study was to compare the adult skeletal morphology of the olfactory regions and the nasal cavities in the frog *R. chensinensis* to those in *Xenopus laevis* and *Bufo regularis*. In addition, based on our morphological findings, we discussed the functional significance of the septomaxilla and the function of the vomeronasal organ in *R. chensinensis*.

2. Materials and Methods

Eight male adult specimens of *R. chensinensis* were collected from the upper stream of the Heihe River, located at the foot of the Tsinling Mountains in Shaanxi, China. The animals were reared in one aquarium of

50 cm × 20 cm × 10 cm with shallow water kept at 20 ± 1 °C, under a 12 h light and 12 h dark photoperiod, and fed with live crickets three times weekly. Animal treatment and care followed the Animal Care Regulations of the Shaanxi Normal University. The animal collection followed the law of the People's Republic of China on the protection of wildlife.

Of the eight specimens, four were euthanized using a 0.1% benzocaine solution and decapitated. The heads were fixed in 4% paraformaldenyde in PBS (pH 7.4) for 4 d at 4 °C. After 4 d of fixation, the heads were decalcified in 2.5% nitric acid for 12–15 d. Dehydration through a graded ethanol series was followed by clearing with xylene. Heads were embedded in paraffin. Serial sections were cut transversely at 8 μm. Specimens were stained with Harris haematoxylin-eosin in order to examine the anatomy of the nasal cavity. All specimens were used in morphological investigations. The sections were observed using a Leica 2 light microscope and illustrated with a Leica Q win V_3 image analysis system.

Another four specimens were double-stained with alcian blue and alizarin red for cartilage and bone (Taylor and Van Dyke, 1985). All specimens are deposited in the College of Life Science, Shaanxi Normal University. Observations and illustrations were made using a Zeiss Discovery V12 stereoscope with an attached Canon 7D digital camera.

3. Results

Generally, the nasal regions are divided into nasal capsules and nasal cavities. The nasal capsules are described below in detail.

3.1 The nasal capsules　The nasal capsules consist of the associated bones and nasal cartilages. Anteriorly, the nasal capsules are separated from each other by the medial, vertical septum nasi (Figure 1 A, B). The septum nasi is always present in serial sections. Ventral to the septum is continuous with the solum nasi. In the ventral of the solum nasi, the cartilago prenasalis inferior projects anteroventrally as a slender process. Dorsolaterally, the anterolateral part of each capsule is supported with the cartilago alaris, a cup-shaped structure, which supports the main part of the outer rim of the apertura nasalis externa. However, it is not connected with the olfactory capsules directly. The glandula intermaxillaris, the largest gland of the nasal region, occupies the prenasal and subnasal spaces. The gland consists of a big, simple mass of secreting tubules mixed with a lot of collecting ducts, all bound together by connective tissue fibers (Figure 1 A).

Posteriorly, the premaxillae are tooth-bearing bones located on the anterior margin of the maxillae. They consist of the partes palatina, facialis and dentalis (Figure 1 C, D). The premaxillae provide bony protection for the anterior portion of the nasal capsules and play an important role in closing of the apertura nasalis externa. The maxillae are also tooth-bearing bones located on the lateral sides of the maxillary arcade, behind the premaxillae (Figure 4 A). The function of the maxillae is to provide bony protection for the ventrolateral aspect of the nasal capsules. Between the premaxilla and maxilla, the lateral edge of the solum nasi curves ventrolaterally into a deep, cartilaginous structure, the crista subnasalis (Figure 1 F). The crista subnasalis projects forwards as a remarkable anterior process.

The septomaxillae, the only bony elements stretching into the nasal capsules, are the most complex bones (Figure 2 B). Each septomaxilla is spiral in shape, with the anterior ramus being more ventral than the posterior one. Moreover, the anterior ramus is more slender than the posterior one (Figure 4 C). The basal portion of the septomaxilla is connected by a thin sheet between the cartilago alaris and crista intermedia.

The upper margin of the septum nasi is curved laterally to form the tectum nasi. Each tectum nasi forms a short cartilaginous roof for the part of the cavum principale (Figure 2 A, B). The tectum nasi stretches ventrolaterally to form the crista intermedia. The crista intermedia can be divided into two laminae: a lamina superior and a lamina inferior. The lamina superior is gradually reduced in width and forms a roof for the cavum medium (Figure 2 C, E).

The nasals, covering the tectum nasi, are bony elements and meet on the medial plane (Figure 3 B, D). The nasals are broader posteriorly than anteriorly (Figure 4 B) and extend from the level of the apertura nasalis externa to the level behind of the choana. However, they do not connect with the premaxillae or the maxillae.

The glandula nasalis medialis is considerable and completely capsular. The gland consists of several minute secreting tubules nearly completely filling the space among the septum nasi, crista intermedia and solum nasi. It extends anteriorly beyond the cavum principale, and posteriorly to a level slightly in front of the choana (Figure 3 A).

Posteriorly, the lateral wall of nasal capsule is mainly formed by a broad cartilaginous element, the planum terminale (Figure 3 D). Medially, the vomers are fixed onto the edge of the solum nasi, the two being attached to each other by fibers (Figure 3 D, F). The vomers are skeletal elements that lie ventral to the olfactory region
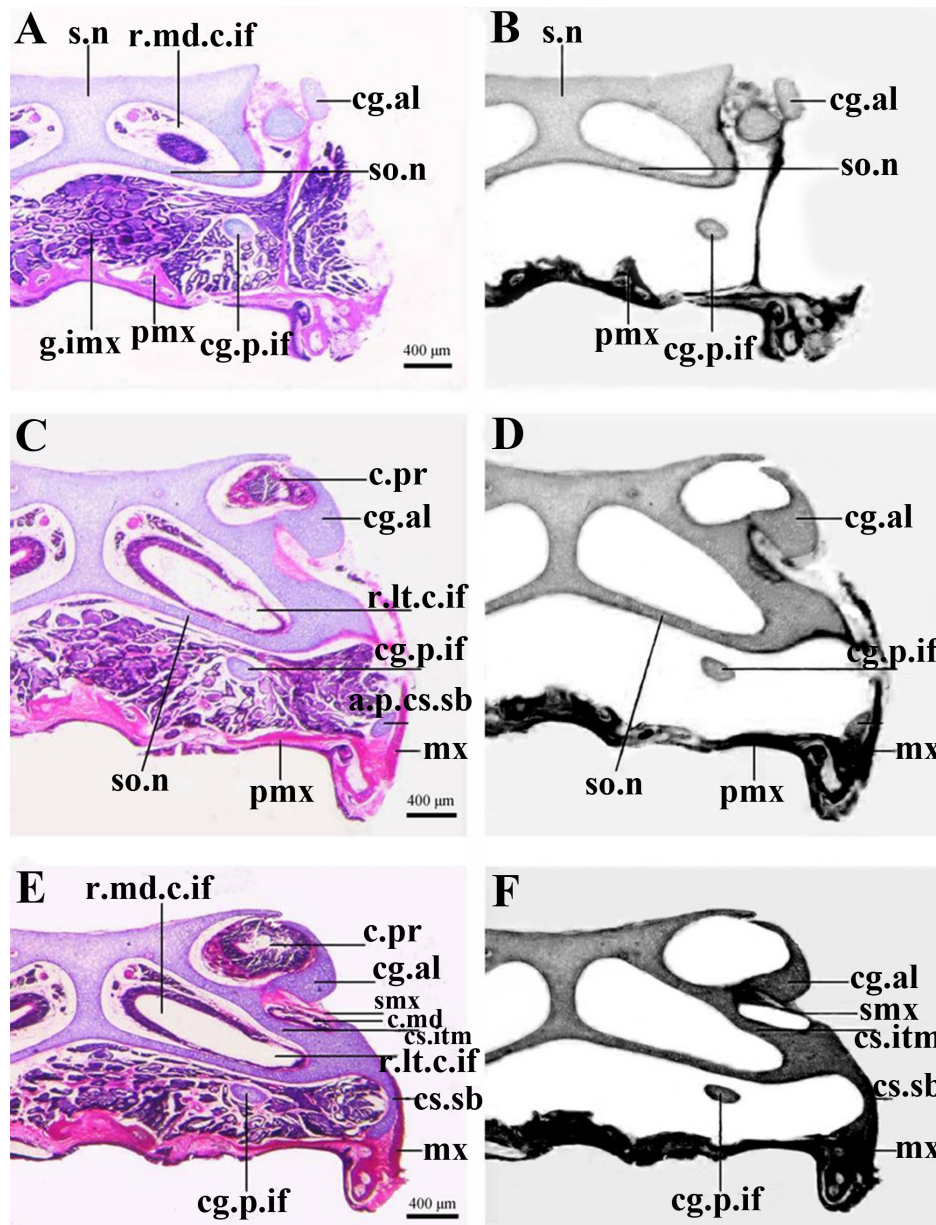
Figure 1 Consecutive cross-sections of the olfactory region of adult *R. chensinensis*. A, B: Sections at the level of the cartilago alaris; C, D: Premaxilla and maxilla; E, F: Crista subnasalis. Abbreviations: a.p.cs.sb. short for anterior process of crista subnasalis; cg.al. for cartilago alaris; cg.p.if. for cartilago prenasalis inferior; c.md. for cavum medium; c.pr. for cavum principale; cs.itm. for crista intermedia; cs.sb. for crista subnasalis; g.imx. for glandula intermaxillaris; mx. for maxilla; pmx. for premaxilla; r.It.c.if. for recessus lateralis of cavum inferius; r.md.c.if. for recessus medialis of cavum inferius; smx. for septomaxilla; s.n. for septum nasi; and so.n. for solum nasi.

and provide ventral support for the nasal capsules. In *R. chensinensis*, the bones underlie the nasal (Figure 4 A). Each vomer is composed of five obvious processes, that is, the anterior, prechoanal, postchoanal, dentigerous, and posteromedial processes. The anterior process of the vomer is rectangular and extends anterolaterally to the level of the premaxilla-maxilla connection. The prechoanal and postchoanal processes extend laterally from the level of the anterior process and their branches extend to the level of the dentigerous process. In the area of the branches, the lateral margin of the prechoanal processes provides support to the eminentia olfactoria. The prechoanal process is short and forms the anterior margin of the choana. Whereas, the postchoanal process is long and its lateral edge reaches the level of the planum triangulare. Besides, the postchoanal process forms the main posterior margin of the choana. The dentigerous process lies medial to the choana and bears the teeth. The

posteromedial process is short and extends posteriorly beyond the level of the dentigerous process (Figure 4 A). At the back of the choana, the septum nasi is gradually ossified. The solum nasi as well as the pars plana nasi curves ventrolaterally into the planum antorbitale (Figure 3 H).

**3.2 The nasal cavities** The nasal cavities consist of three main chambers: the cavum principale, cavum medium and cavum inferius (Figure 2 A). The cavum principale is the largest and most dorsal cavum which also contains the main olfactory organ. The dorsal wall of the cavum principale is mainly formed by the tectum nasi and the floor is formed by the crista intermedia. The anterior region of the cavum principale leads the outside through the apertura nasalis externa (Figure 2 E). Posteriorly, it connects to the oral cavity via the choana (Figure 3 E). Anterior to the choanal region, the floor of the posterior
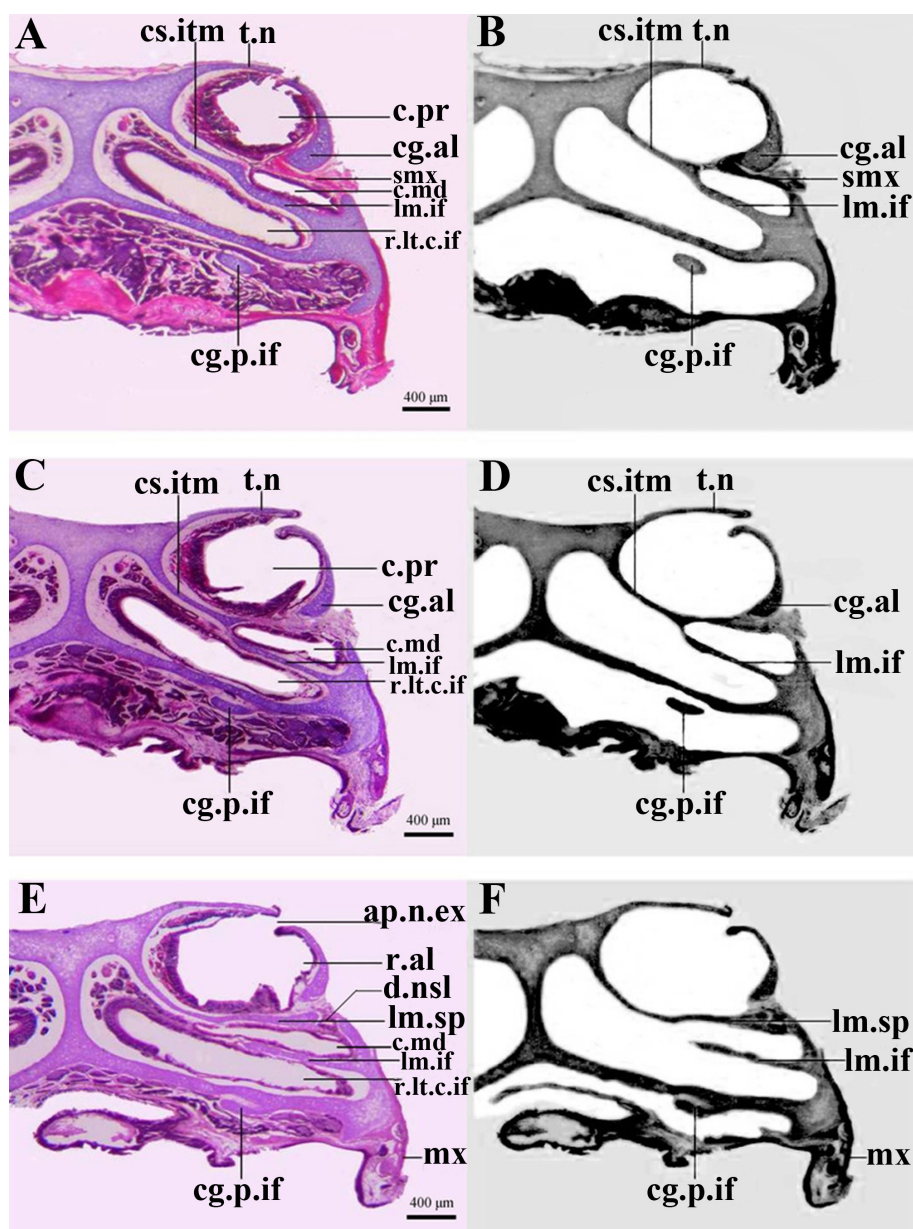

Figure 2 Consecutive cross-sections of the olfactory region of adult *R. chensinensis*. A, B: Sections at the level of the tectum nasi and septomaxilla; C, D: Apertura nasalis externa; E, F: Laminae superior and inferior of the crista intermedia. Abbreviations: ap.n.ex. short for apertura nasalis externa; cg.al. for cartilago alaris; cg.p.if. for cartilago prenasalis inferior; c.md. for cavum medium; c.pr. for cavum principale; cs.itm. for crista intermedia; d.nsl. for nasolacrimal duct; lm.if. for lamina inferior of crista intermedia; lm.sp. for lamina superior of crista intermedia; mx. for maxilla; r.al. for recessus alaris; r.lt.c.if. for recessus lateralis of cavum inferius; smx. for septomaxilla; and t.n. for tectum nasi.

half of the cavum principale is elevated by the eminentia olfactoria. The eminentia olfactoria greatly reduces the space of this cavum, but increases its olfactory surface (Figure 3 E).

Along the ventral floor of the cavum principale is the cavum medium (Figure 2 A, C). The cavum medium is situated between the cavum principale and cavum inferius. The laminae superior and inferior of the crista
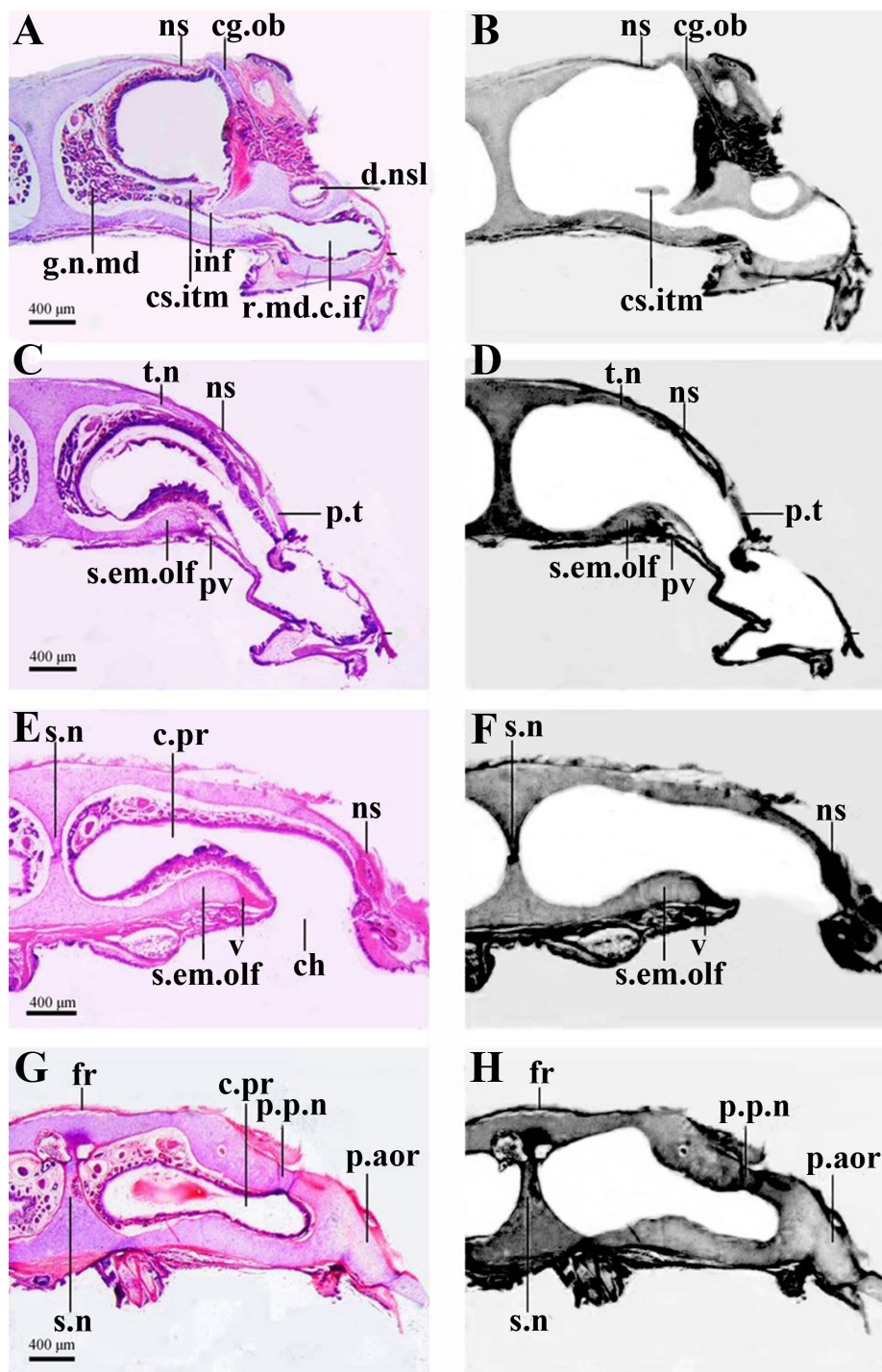
Figure 3 Consecutive cross-sections of the olfactory region of adult *R. chensinensis*. A, B: Sections at the level of the nasal; C, D: Planum terminale; E, F: Vomer and choana; G, H: Posterior margin of choana. Only the right half of each section is shown. Abbreviations: cg.ob. short for cartilago obliqua; ch. for choana; c.pr. for cavum principale; cs.itm. for crista intermedia; d.nsl. for nasolacrimal duct; fr. for frontal; g.n.md. for glandula nasalis medialis; inf. for infundibulum; ns. for nasal; p.aor. for planum antorbitale; p.p.n. for pars plana nasi; p.t. for planum terminale; v. for vomer; r.md.c.if. for recessus medialis of cavum inferius; s.em.olf. for skeletal element of eminentia olfactoria; s.n. for septum nasi; and t.n. for tectum nasi.
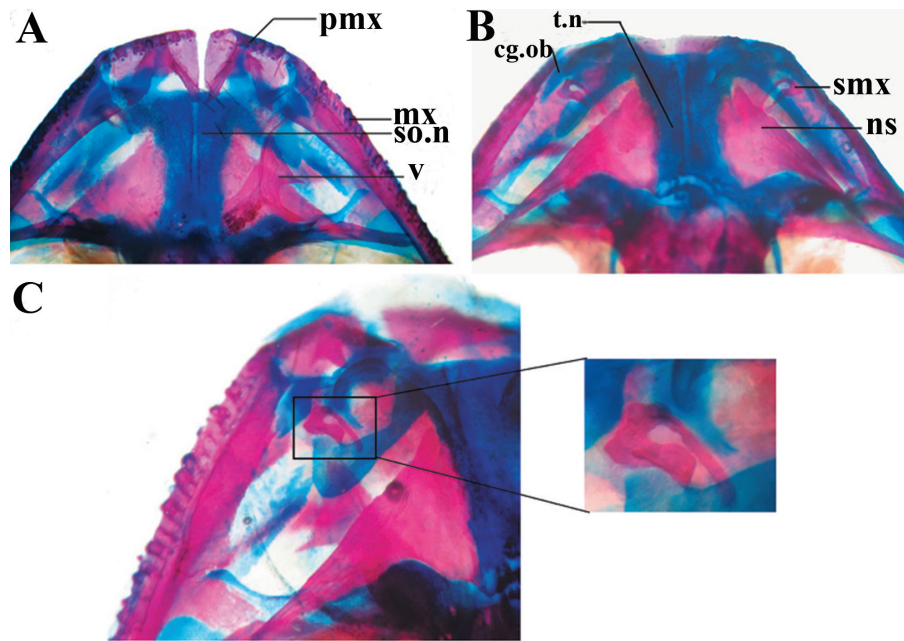
Figure 4 Double-stained cartilage and bone with alcian blue and alizarin red. A: Ventral view of the skull showing the vomer (the left vomer has been removed); B: Dorsal view of the skull showing the nasal; C: Dorsal view of the anterior part of the frog's head showing the septomaxilla. The septomaxilla is shown in more detail on the right micrograph. Abbreviations: cg.ob. short for cartilago obliqua; mx. for maxilla; ns. for nasal; pmx. for premaxilla; v. for vomer; smx. for septomaxilla; so.n. for solum nasi; and t.n. for tectum nasi.

intermedia surround the cavum medium. Posteriorly, the cavum medium gradually fades out and continues with the lower component of the nasolacrimal duct.

Below the cavum medium is the cavum inferius (Figure 2 E). The cavum inferius is larger than the cavum medium and contains the vomeronasal organ. In *R. chensinensis*, the vomeronasal organ is located on the ventromedial side of the nasal cavity, the recessus medialis of the cavum inferius. The lamina inferior of the crista intermedia forms a roof for the cavum inferius and the solum nasi acts as a floor. Just in front of the choana, the recessus medialis of the cavum inferius extends into two branches: a dorsal branch connecting to the cavum principale through the infundibulum and a ventrolateral branch joining the recessus lateralis of the cavum inferius (Figure 3 A). At the back of the choanal region, the recessus laterralis gradually decreases in size (Figure 3 C).

4. Discussion

4.1 The eminentia olfactoria   The eminentia olfactoria is a small bump on the floor of the cavum principale and is fixed onto the lateral edge of the vomer. Helling (1938) thought that the eminentia olfactoria was well developed in the anurans of which the adults are mainly terrestrial and that the eminentia olfactoria was missing in extremely aquatic species. For example, *X. laevis* has no remarkable

eminentia olfactoria (Paterson, 1939) for it is a purely aquatic frog. In comparison with *X. laevis,* the eminentia olfactoria of fossorial species (such as *Spea bombifrons*) and terrestrial species (*B. regularis* Reuss) are well developed (Michael, 1961; Jurgens, 1971; Pugener and Maglia, 2007). *B. regularis* is generally considered to be a savanna species and its adults spend more time on land. Our results showed that the eminentia olfactoria of *R. chensinensis* is present and is not developed in the posterior portion of the cavum principale. The adults of *R. chensinensis* live mainly in wood regions, and they also occur in hill forests, particularly those near streams and ponds (Fei *et al.*, 2006). The eminentia olfactoria can increase the contact surface with the air in order to facilitate air movement. The eminentia olfactoria of the cavum principale in frogs closes the apertura nasalis externa by the submentalis muscle and draws air-borne odorants into the olfactory cavities during lung inflation (Reiss and Eisthen, 2008). Therefore, the origin of the eminentia olfactoria in the olfactory cavity may be an adaption to the terrestrial environment.

4.2 The nasolacrimal duct   In extant tetrapods, the nasolacrimal duct is a tubular channel that carries mucus from the Harderian gland to the nasal cavity. Nowack *et al.* (2010) concluded that there were two main types of nasolacrimal ducts: 1) The opening of the nasolacrimal

duct shows a close proximity to the apertura nasalis externa and is a great distance to the vomeronasal organ; and 2) the opening region of the nasolacrimal duct is situated in caudal part of the apertura nasalis externa and closer to the vomeronasal organ. Our results indicate that the opening of the nasolacrimal duct is close to the apertura nasalis externa in *R. chensinensis*.

Several morphological observations suggest that the nasolacrimal duct may have a functional association with the vomeronasal organ in these taxa. For example, Hillenius *et al.* (2001) applied India ink to the surface of the frog's eye where the India ink was ultimately found in the vomeronasal organ. According to their result, the chemical stimuli may be absorbed in the cornea of the eye, and then were carried with the tear into the vomeronasal organ by the nasolacrimal duct, so as to activate the vomeronasal organ in anurans. Nevertheless, Nowack *et al.* (2010) discovered that stimuli reaching the vomeronasal organ are more likely to be collected through the apertura nasalis externa than via the eye. The nasolacrimal duct can carry fluid from the Harderian gland into the nasal cavity, and then the fluid lubricates the vomeronasal organ, so as to sense the stimuli. In addition, the nasolacrimal duct of *R. chensinensis* is present during metamorphosis, which correlates with the appearance of the forelimbs (Wang *et al.*, 2008). Conclusively, the presence of the nasolacrimal duct, as a primitive attribute in tetrapods, could mediate a functional interaction between the Harderian gland and the vomeronasal organ on land.

4.3 The septomaxilla The most important bone associated with the olfactory capsules is the septomaxilla. The septomaxilla is a small, isolated bone, which is closely associated with the apertura nasalis externa, and forms an F-shaped region in dorsal view (Jurgens, 1971). Among tetrapods, the septomaxilla is present only in some amphibians and most lepidosaurs. It is absent in turtles, crocodilians, and birds. In mammals, the septomaxilla is generally reduced or lost; it persists only in monotremes (Hillenius, 2000). The septomaxilla usually has two basic types: 1) as an intramembranous bone, which is separated from the adjacent cartilages of the olfactory capsule by connective tissue fibers; and 2) as endochondral ossification, which is fixed to the adjacent cartilages of the olfactory capsule closely, with *Bufo marinus* being a typical example (Michael, 1961). The septomaxilla in *R. chensinensis* shares the membrane origin and intramembranous ossification.

The special shape of the septomaxilla and its remarkable position in the nasal capsules suggest that this bone serves a specialized function. In anurans, the nasolacrimal duct passes through a deep slit in the lateral ramus of the septomaxilla before emerging into the nasal cavity (Trueb and Cannatella, 1982; Hillenius, 2000). Generally, the function of the septomaxilla is to provide support to the anterior end of the nasolacrimal duct (Michael, 1961). The embryonic development of the septomaxilla is highly dependent on the presence of the nasolacrimal duct. In other words, when the nasolacrimal duct is lost, the septomaxilla is also lacking (Jurgens, 1971; Hillenius, 2000). Besides, it seems that there may be a particular correlation between the septomaxilla and nasolacimal duct.

4.4 The function of the vomeronasal organ The vomeronasal organ was first present in amphibians and is considered to have been absent in fishes. Broman (1920) believed that the vomeronasal organ in amphibians is homologous with the olfactory organ in fishes. The olfactory organ arose later as an adaptation to terrestrial life, while the vomeronasal organ sensed water-borne odorants. Døving *et al.* (1993) carried out a vital staining assay where adult frogs were kept in a solution of Cresyl Violet which can produce an intense fluorescence. The result showed that the oral cavity and the epithelium of the cavum principale were almost unstained, whereas the vomeronasal organ could be observed as strongly stained. Therefore, they believed that the vomeronasal organ in the frogs is a water sampling organ. Belanger *et al.* (2009) also insisted that the adult anuran nasal cavity can sample the chemical composition of the both terrestrial and aquatic environments. The cavum principale is used for sampling air-borne odorants, whereas the vomeronasal organ samples water-borne substances.

However, Bertmar (1981) subverted the theory that the vomeronasal organ is a water sampling organ. The vomeronasal organ was present in tetrapods but absent in fish, and among tetrapods it was vestigial or absent in crocodilians, birds, most bats, and marine mammals. Therefore, the author indicated that the vomeronasal organ exists only in tetrapods as an adaptation to the terrestrial environment. Eisthen (2000) suggested that the vomeronasal organ was generally present in aquatic amphibians and did not originate as an adaptation to terrestrial life. According to the above results, the functional importance of the vomeronasal organ is an almost unanswered question. Further studies are needed to investigate the functional implications of the vomeronasal organ in future.

References

Bertmar G. 1981. Evolution of vomeronasal organs in vertebrates. Evolution, 35(2): 359–366

Belanger R. M., Corkum L. D. 2009. Review of aquatic sex pheromones and chemical communication in anurans. J Herpetol, 43(2): 184–191

Broman I. 1920. Das Organon vomero-nasale Jacobsoni-ein wassergeruchsorgan. Anat Hefte, 58(1): 143–191

DØving K. B., Trotier D., Rosin J. F., Holley A. 1993. Functional architecture of the vomeronasal organ of the frog (genus *Rana*). Acta Zool, 74(3): 173–180

Eisthen H. L. 1992. Phylogeny of the vomeronasal system and of receptor cell types in the olfactory and vomeronasal epithelia of vertebrates. Microsc Res Tech, 23(1): 1–21

Eisthen H. L. 1997 Evolution of vertebrate olfactory systems. Brain Behav Evol, 397 (50): 222–233

Eisthen H. L. 2000. Presence of the vomeronasal system in aquatic salamanders. Philos Trans R Soc Lond B Biol Sci, 355(1401): 1209–1213

Fei L., Hu S. Q., Ye C. Y., Huang Y. Z. 2006. Fauna Sinica, Amphibia, Vol.1. General Accounts of Amphibia, Gymnophiona and Urodela. Beijing: Science Press, 59–63

Helling H. 1938. Das Geruchesorgan der Anuren, vergleichend-morphologisch betrachtet. Ger J Anat, 108: 587–643

Higgins G. M. 1920. The nasal organ in Amphibia. Illinois Biol Monogr, 6: 49–59

Hillenius W. J. 2000. Septomaxilla of nonmammalian synapsids: Soft-tissue correlates and a new functional interpretation. J Morphol, 245(1): 29–50

Hillenius W. J., Watrobski L. K., Rehorek S. J. 2001. Passage of tear duct fluids through the nasal cavity of frogs. J Herpetol, 35(4): 701–704

Jurgens J. D. 1971. The morphology of the nasal region of Amphibia and its bearing of the phylogeny of the group. Ann Univ Stellenbosch, 46(2): 136–146

Michael M. I. 1961. The adult morphology of the olfactory organs of the Egyptian Toad, *Bufo regularis* Reuss. J Morphol, 109(1): 1–17

Nowack C., Wöhrmann-Repenning A. 2010. The nasolacrimal duct of anuran amphibians: Suggestions on its functional role in vomeronasal perception. J Anat, 216(4): 510–517

Paterson N. F. 1939. The head of *Xenopus laevis*. Q J Microsc Sci, s2–81(322): 161–232

Pugener L. A., Maglia A. M. 2007. Skeletal morphology and development of the olfactory region of *Spea* (Anura: Scaphiopodidae). J Anat, 211(6): 754–768

Reiss J. O., Eisthen H. L. 2008. Chemical senses: Comparative anatomy and physiology of chemical senses in amphibians. In Thewissen H., Nummular S. (Eds.), Sensory Evolution on the Threshold: Adaptations in Secondarily Aquatic Vertebrates. Berkeley: University of California Press, 43–63

Taylor W. R., Van Dyke G. C. 1985. Revised procedure for staining and clearing small fishes and other vertebrates for bone and cartilage study. Cybium, 9(2): 107–119

Taniguchi K., Saito S., Taniguchi K. 2011. Phylogenic outline of the olfactory system in vertebrates. J Vet Med Sci, 73(2): 139–147

Trueb L., Cannatella D. C. 1982. The cranial osteology and hyolaryngeal apparatus of *Rhinophrynus dorsalis* (Anura: Rhynophryni-dae) with comparisons to recent pipid frogs. J Morphol, 171: 11–40

Wang H. Y., Zhao H. F., Tai F. D., Zhang Y. H. 2008. Postembryonic development of the olfactory and vomeronasal organs in the frog *Rana chensinensis*. Zool Sci, 25(5): 503–508